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Full Length Research Paper

In vitro multiplication of the important medicinal plant, harmal (*Rhazya stricta* Decne)

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An improved protocol for successful micropropagation of the important medicinal plant 'harmal' (*Rhazya stricta* Decne) has been developed. Nodal segments containing axillary buds were surface sterilized and inoculated aseptically on culture medium. The effect of the combinations of 6-benzylaminopurine (BAP) and kinetin (kin) on shoots initiation, proliferating and elongation of the explants, as well as the effect of Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA) on root formation were studied. The highest break of axillary buds was achieved in MS medium supplemented with 2 mg/L BAP and 1 mg/L kin as 72% of explants showed shoot initiation from the auxiliary buds. When the initiated shoots were sub-cultured in the same medium constitution, the best multiplication frequency was obtained. The proliferated shoot clusters showed higher elongation rate (73%) when it cultured in MS medium supplemented with 3 mg/L BAP and 1 mg/L kin. The best rooting frequency was achieved on full strength MS medium containing 3 mg/L IBA.

Key words: Medicinal plants, *Rhazya stricta* Decne, nodal segments, axiliary buds, micropropation, multiplication.

INTRODUCTION

Rhazya stricta Decne of the Apocynaceae family is an evergreen dwarf shrub widely distributed in the sandy plains of Saudi Arabia and comparable habitats throughout the world (Chaudhary and Al- Jowaid, 1999). It is abundantly found in Western Asia from Yemen to Saudi Arabia, to the North West Province of India and various regions of Pakistan. This plant species is one of the famous plants characterizing the habitat of Saudi Arabia and is considered one of the most important medicinal plants that grow in the most desert areas in the Arabian Peninsula. The vernacular name of *R. stricta* is 'harmal', also applied to another noxious weed, *Peganum harmala* (family Zygophyllaceae). However, the later species is widely distributed in the northern regions of Saudi Arabia whereas *R. stricta* northern range of distribution does not extend that far (Chaudhary, 2000). *R. stricta* is perennial, cushion shaped herb, branched from the base to height of 115 cm. and spread to about 235 cm and has adventitious roots spread over a large area in the soil. The phonological aspects show that there is a regular life cycle which starts from the perennial plant or the seed after the seasonal rains during November, the flowering starts at the beginning of January and the fruiting at the middle of it. Irregular flowering and fruiting periods were recorded from May to June (Chaudhary, 2000).

The economic value of *R. stricta* can be approached from two contradicting views. First, as an invasive weed into rangelands that caused retrogression to their ecological condition, thus ought to be controlled; and second as a valuable native medicinal plant that needs to be preserved. *R. stricta* is extensively used in folk

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medicine. Several studies proved its medical benefits as it contains milky sap with toxic compounds and many other materials that can be used in the treatment of certain diseases especially as an antimicrobial (Atta et al., 1991; Saeed et al., 1993; Bashir et al., 1994; Ali, 1998). Its infusion is a good tonic with peculiar bitter taste. Phytochemical analysis has identified more than 100 alkaloids (Gilani et al., 2007). These alkaloids have several pharmacological properties. In South Asia and Middle East countries, *R. stricta* is used traditionally as an indigenous medicinal herbal drug in treatment of different types of diseases such as skin diseases, stomach diseases and antihypertensive (Mukhopadhyay et al., 1981). The leaves, flowers and fruit are also used in joint infections and for cancer (Rahman and Qureshi, 1990). It is also used for throat sour, in fever, general debility and as curative for chronic rheumatism and tumor.

Tissue culturing of medicinal plants is widely used to produce active compounds for herbal and pharmaceutical industries. Advances in plant tissue culture will enable rapid multiplication and sustainable use of medicinal plants for future generations. Comparing with traditional methods of producing medicinal plant, *in vitro* micropropagation have many advantages such as the independent of seasonal variation, mass production, identification and production of clones with desired characteristics, conservation of threatened plant species, production of new and improved genetically engineered plant, preservation of genetic material by cryopreservation and production of secondary metabolites. Micropropagation of Several medicinal plants has been reported. Few studies reported the micropropagation of *P. harmala* (Saini and Jaiwal, 2000; Ehsanpour and Sa-Adat, 2002) and other studies concerned on the production of secondary metabolites (Reinhard et al., 1968; Nettleship and Slaytor, 1974; Sasse et al., 1987; Berlin and Sasse, 1988; Courtois et al., 1988; Berlin, 1989, 1999) through callus formation. To date, to our knowledge, no *in vitro* multiplication study of. *R. stricta* Decne has been reported. Thus in the present study, we present an improved protocol for *in vitro* multiplication of this important medicinal plant.

MATERIALS AND METHODS

Plant material, explants source and sterilization

Nodal contains axillary buds of *R. stricta* were collected from mature plants grown in the open field at Ymanyiah region, Taif governorate, KSA. The source tissues were washed first under running tap water to remove the superficial dust followed by a detergent (3 min). After a thorough wash in sterile water, the source tissues were surface sterilized 20% with commercial chloride for 7 to 10 min. Following repeated washes with sterile water (three washes, 5 min each), node segments containing and shoot tips were cut into appropriate sizes (1 to 1.5 cm) and cultured on sterile medium.

Medium and culture conditions

The explants were placed on solid basal MS (Murashige and Skoog, 1962) medium gelled with 0.8% (w/v) agar and supplemented with different concentrations and combinations of (BAP) 1, 2 and 3 mg/L with and without 1 mg/L kinetin (kin) for shoot proliferation and multiplication. The pH was adjusted to 5.6 using 0.1 N NaOH or 0.1 N HCl before autoclaving (121°C, 20 min). The shoots were maintained by regular subcultures at 4-week intervals on fresh medium with the same compositions. For root induction, *in vitro* micro-shoots (1 to 2 cm length) were excised aseptically and implanted on solidified full and half strength MS basal medium without growth regulators or supplemented with different concentrations of IAA 0 and 1 mg/L, and IBA 1, 2 and 3 mg/L. All the cultures were incubated at 25 ± 2°C under a 16 h photoperiod with cool and white fluorescent lamps (3000 lux). Rooted explants were planted in pots containing a sterile soil and kept in the green house for acclimatization.

RESULTS

The nodal explants were incubated on MS medium without growth regulator or fortified with different concentrations of BAP (1.0, 2.0 and 3.0 mg/L) alone or with 1 mg/L kin. The results are summarized in Table 1. The axillary bud break was observed in all medium used after two weeks. When BAP was used alone, increase of its concentration resulted in a slightly gradual increase in the shoot formation frequency with the highest record of 40% at the concentration of 3 mg/L. However, the best frequency of break of axillary buds was achieved in MS medium supplemented with 2 mg/L BAP and 1 mg/L kin. At this concentration, the best percentage of explants (72.0%) showed shoot initiation comparing with other concentrations used (Table 1).

After four weeks, emerged multiple shoots directly from axillary nodes of the cultured explants were recorded in all concentrations of BAP alone or with combination with kin (Table 1). Medium supplemented with 2 mg/L BAP and 1 mg/L kin were found to be the ideal concentration to induce maximum shoots (15) per nodal explant (Table 1). However, the shoots elongation was recorded in all the medium-used; its best frequency was achieved in MS medium supplemented with 3 mg/L BAP and 1 mg/L kin.

For rhizogenesis, the *in vitro* regenerated shoots were excised aseptically and implanted on full and half strength MS medium without or with growth regulators (IAA and IBA). Excised shoots failed to develop roots on both full and half strength MS medium without growth regulators. Rooting was recorded only when full strength MS (major and minor salts and vitamins) medium was used in combination with different concentrations of IBA and IAA (Table 2), although root formation, accompanied with callusing at the shoot base in all concentrations (Figure 1E). While, using half strength MS medium resulted in general weakness and yellowish coloration of the shoots with no-root formation (data not shown). Induction of roots was noticed after 19 days of implantation in all medium. Rooting frequency of 40 to

Table 1. Effects of different concentration and combination of the cytokinins on *in vitro* shoot proliferation from nodal segments explants of *R. stricta*.

Supplement (mg/L)		% of explants showing shoot proliferation	No. of shoots per explants (mean ± SE)	Frequency of shoot elongation (%)
BAP	Kin			
0.0	0.0	0.0	0.0 ± 0.0	0.0
1.0	0.0	28.0	6 .0 ± 0.15	46.6
2.0	0.0	30.0	7.0 ± 0.17	36.7
3.0	0.0	40.0	9.0 ± 0.09	33.3
1.0	1.0	24.0	4.0 ± 0.11	40.0
2.0	1.0	72.0	15.0 ± 0.29	50.0
3.0	1.0	44.0	11.0 ± 0.23	73.3

Each treatment consisted of 3 replications in which 10 to 12 explants were used.

Table 2. Effects of different concentration and combination of IBA and IAA on rooting of *R. stricta* under *in vitro* conditions.

Supplement (mg/L)		Treatment period (day)	Frequency of rooting (%)	Number of roots per shoot (mean ± SE)	Root length (cm) (mean ± SE)
IBA	IAA				
0.0	0.0	21	0.0	0.0 ± 0.0	0.0 ± 0.0
1.0	1.0	21	40.0	1.0 ± 0.09	1.4 ± 0.06
2.0	1.0	21	66.7	2 .0 ± 0.11	1.7 ± 0.07
3.0	0.0	21	86.7	4.0 ± 0.13	4.4 ± 0. 06

Each treatment consisted of 3 replications in which 10 to 12 explants were used.

66.7% was obtained on the first two concentrations of IBA and IAA, respectively ($p < 0.01$; Table 2). However, the concentration of 3 mg/L of IBA was found optimal with best rooting formation (86.7%), maximum number of 4.0 roots and the longest mean root length of 4.4 cm.

DISCUSSION

The *in vitro*-propagated medicinal plants furnish a ready source of uniform, sterile, and compatible plant material for biochemical characterization and identification of active constituents (Wakhlu and Bajwa, 1986; Miura et al., 1987). In addition, compounds from tissue cultures may be more easily purified because of simple extraction procedures and absence of significant amounts of pigments, thus possibly reducing the production and processing costs (Chang et al., 1994).

In the present study, a protocol for mass production of the important medicinal plant *R. stricta* Decne, using the nodal explants was developed (Figure 1). The medium devoid of growth regulators failed to induce the formation of shoot buds (data not shown). Similarly, no shoot buds developed in *Peganum harmala* (Saini and Jaiwal, 2000) and *Crataeva nurvala* (Walia et al., 2003) on MS basal medium. It has been suggested that the growth regulators applied externally during *in vitro* studies might disturb the internal polarity and change the genetically programmed physiology of explants resulting in organogenesis.

The cytokinin BAP was used for the axillary bud break and shoot multiplication. BAP was reported as efficient growth regulator for shoot multiplication in *Chlorophytum borivilianum* (Sharma and Mohan, 2006) and *Cyphomandra betacea* (Chakraborty and Roy, 2006). It was speculated that shoot apices and axillary buds are most likely to contain different levels of internal auxin, cytokinin and abscisic acid (ABA) concentrations which influenced the level of adventitious shoot regeneration from first axillary buds (Khalid et al., 2005). Moreover, it was reported that MS medium containing combination of 1.5 mg/L IBA + 0.5 mg/L kin) was the best for shoot initiation and multiplication in several species such as *Fragaria indica* Andr (Bhatt and Dhar, 2000), papaya (Cononer and Litz, 1978) and *Eucalyptus grandis* (Teixetra and Dasilva, 1990) as well as *Stevia rebaudiana* Bertoni (Ahmed et al., 2007).

The development of axillary shoots was accompanied by basal callusing of the explants (Figure 1B). However, this remains undifferentiated. Same type of observations were made by Dhawan and Bhojwani (1985), Kackar et al. (1991), Nandwani and Ramawat (1991) and (Saini and Jaiwal, 2000) working with *Leucaena leucocephala*, *Prosopis cineraria*, *Prosopis juliflora* and *Peganum harmala*, respectively. Marks and Simpson (1994) suggested that this callus formation may be due to the

Figure 1. Micropropagation of Harmal *(Rhazya stricta* Decne) using nodal segments. A: nodal segments containing axillary buds placed in MS medium. B: Initiation of axillary shoot and basal callusing of the explants. C: Multiplication of axillary shoots. D, E and F: Elongation of excised shoots. G: Rooting stage. H; Acclimatization.

action of accumulated auxin at the basal cut ends, which stimulates cell proliferation especially in the presence of cytokinins. According to Preece et al. (1991), the formation of callus at basal cut ends of nod explants on cytokinin enriched medium is frequent in species with strong apical dominance.

To our knowledge, the recent developed protocol is the first for *in vitro* multiplication of the *R. stricta* Decne. The protocol will be evaluated by molecular markers tools to evaluate the genetic fidelity during micropropagation to determine the possibility for using it for rapid multiplication to produce active compounds for herbal and pharmaceutical industries.

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